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PREPARATION OF HYDROPHILIC COPOLYMERS IN BEAD FORM AS CARRIERS IN AFFINITY CHROMATOGRAPHY

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SUMMARY

In order to prepare gels for affinity chromatography, copolymers were prepared from agarose, N,N'-methylenebisacrylamide, in some instances from acrylamide, and an acrylic monomer carrying a carboxylic group well separated from the double bond. The acrylic monomers used were 6-acrylamidohexanoic acid and N-methacryloylglycylglycine, whose copolymerization provided carriers that possess either hydrophobic or hydrophilic spacer arms.

The amount of carboxyl groups on the gels obtained is proportional to the concentration of acid monomer in the initial mixture. It is therefore possible to obtain gels that have the number of carboxyl groups required, contrary to the more restrictive and less reproducible traditional methods, which involve chemical modifications of various polymers, mostly of natural origin (polysaccharides).

INTRODUCTION

Affinity chromatography is one of the major biochemical techniques of separation on gels and has become a basic method for the purification of many natural substances, especially enzymes[1].

Affinity chromatography is based on the selective adsorption of proteins on effectors, or of the complementary proteins, immobilized on an insoluble macromolecular carrier. The carrier–ligand conjugate is generally used as a stationary phase in a chromatographic column. In practice, when a crude mixture containing the protein to be purified is placed in the column, the protein forms a complex by affinity with the immobilized ligand of the stationary phase, where it is thus retained. The contaminating proteins, on the contrary, continue to migrate and leave the column. The elution of the retained protein is then effected by destroying the complex in various way, *e.g.*, by percolating a solution of an effector of the protein, by modi-

fying the pH[2,3], by increasing the ionic strength[4] or by the combination of these methods[5].

The carrier must have some special features[6], such as a neutral hydrophilic character and good porosity with regard to the proteins, and it must have the shape of beads between 60 and 200 μm in size. Carriers that satisfy these requirements are polyacrylamide gels (Bio-Gel P), dextran gels (Sephadex G) and the agarose gels (Sepharose), and mixed polyacrylamide–agarose gels (Ultrogel AcA; LKB, Bromma, Sweden).

The methods that make it possible to fix the ligand involve bifunctional agents such as cyanogen bromide, which is the most widely used activation reagent with polyosidic carriers[7,8]. At first, under the action of cyanogen bromide, imidocarbonate groups are formed, which react with the amine function of the ligand to be immobilized. Measurement of the pK of the reaction products shows that the formation of an isourea bond is the most likely[9–11]. Although this process sometimes gives satisfactory results[12–14], the ligand must be fixed at a certain distance from the matrix[15], which can be achieved by various methods, particularly those proposed by Cuatrecasas[16,17]. The most convenient means of introducing a spacer arm is probably to make the carrier react with the ω-amino group of a compound such as $H_2N-(CH_2)_n-X$, in which X is generally a carboxyl group and more rarely an amino group. One thus obtains a gel carrying functionalized side-chains on which immobilization of the ligand is effected by means of a water-soluble carbodiimide[18,19].

All of these reactions are carried out on insoluble polymers and their yields are generally poor. Moreover, owing to the limited proportion ($<10\%$) of dry material in the hydrated gel, the amount of ligand fixed is small (generally 2–10 μmole per millilitre of gel). The fixation of larger amounts of ligand, which may be made necessary in some instances, can be achieved only at the cost of additional difficulties resulting, for instance, from the use of large amounts of dangerous activating reagents, especially cyanogen bromide. In this way, one can increase considerably the rate of fixation on agarose of β-aminoethyltrimethylammonium bromide hydrobromide, provided that up to 0.6 g of cyanogen bromide per gram of carrier is used[20]. Porath and Sunberg[21] proposed to increase the fixation capacity of polyosidic carriers by multiplying the number of free hydroxyl groups by means of preliminary chemical treatment. However, in general, and as can be expected, the activation of a polymer by di- or poly-functional reagents, involves ill-defined chemistry and is hardly controllable and reproducible, and hence is unreliable, as was observed with immobilized enzymes[22].

However, the above drawbacks can be avoided by using a synthetic carrier prepared by the copolymerization of an appropriate monomer, carrying a sufficiently long chain and ending with an appropriate functional group. Following this approach, Koch-Light (Colnbrook, Great Britain) were able to introduce a whole range of functionalized acrylic resins (Enzacryls) that are particularly suitable for the immobilization of enzymes[23–25]. On the other hand, Wilchek[26] and Wilchek and Miron[27] prepared polyacrylhydrazidoagarose for affinity chromatography, and discussed in detail the advantages arising from the combination of the properties of agarose and polyacrylamide gels.

In this work, we followed similar lines, but our aim was to prepare copolymers carrying side-chains ending with carboxyl groups, intended for affinity chromato-

graphy. We first describe the synthesis of the monomers and then we consider the conditions of copolymerization that allowed us to obtain hydrophilic gels in the form of microspheres with good mechanical properties.

EXPERIMENTAL

Chemicals

Indubiose A-37 (agarose) was supplied by L'Industrie Biologique Française (Gennevilliers, France). N,N,N',N'-Tetramethylethylenediamine (TEMED) was obtained from Sigma (St. Louis, Mo., U.S.A.). 6-Aminohexanoic acid, glycylglycine and N,N'-methylenebisacrylamide (BIS) were provided by Aldrich (Milwaukee, Wisc., U.S.A.).

6-Acrylamidohexanoic acid (Ac.N-A-6-H)

This monomer, mentioned earlier in a German patent[28], was prepared according to the reaction

$$CH_2 = CH\text{–}COCl + H_2N\text{–}(CH_2)_5\text{–}CO_2H \xrightarrow[(2)\ HCl]{(1)\ NaOH} CH_2 = CH\text{–}CO\text{–}NH\text{–}(CH_2)_5\text{–}CO_2H$$

(Ac.N-A-6-H)

Dissolve 26.4 g of 6-aminohexanoic acid in 200 ml of 2 *N* sodium hydroxide solution. Cool the solution to 0° and in 30 min, with magnetic stirring, add 18 g of distilled acryloyl chloride. After stirring for 1 h, acidify to pH 3 with 10% hydrochloric acid. Recover by filtration the white precipitate obtained and recrystallize it in as little boiling ethyl acetate as possible. On cooling, the solution leaves 26.6 g (yield of about 72%) of white crystals, m.p. 84–89°. The following spectral data confirmed the structure proposed for this monomer: IR spectrum (Nujol), 1682 and 1634 cm^{-1} (CO groups); NMR spectrum (DMSO-d6), δ 1.36 ppm [$-(CH_2)_3-$], δ 2.13 ppm ($-CH_2-CO-$), δ 3.1 ppm ($>N-CH_2-$), δ 5.47, 6.04 and 6.13 ppm (vinyl protons).

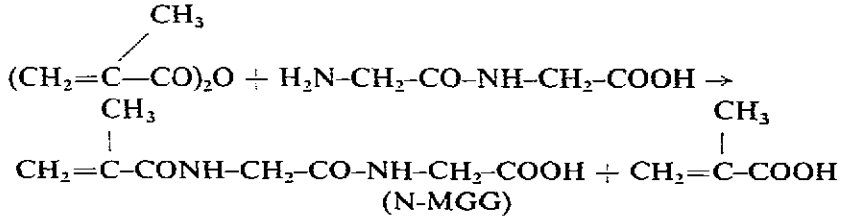
N-Methacryloylglycylglycine (N-MGG)

Earlier work such as that by Smith[29], and more recent work such as that by Batz and Koldehoff[30], involved the esters of N-methacryloylglycylglycine and its acrylic analogue, and the preparation of the free acids was not mentioned. Smith even specified that the saponification of the ethyl ester proceeds with a cyclization or a polymerization, which excludes any possibility of isolating the free acid.

We decided to synthesize this compound because it was likely to be water-soluble. Indeed, the copolymerization of this compound, as well as that of 6-acrylamidohexanoic acid, must be carried out in an aqueous medium.

In order to prepare this acrylic derivative, we discarded the traditional methods in which water is used as the only reaction solvent, as we expected that the recovery of the monomer would be difficult under such conditions. Our synthesis, which is based on a method described by Brain *et al.*[31] for other compounds, consists in

adding an acetone solution of methacrylic anhydride to an aqueous solution of glycylglycine, according to the following reaction:



Dissolve 24.4 g of glycylglycine in 100 ml of distilled water and heat the solution to 50°. Separately dissolve 34 g of methacrylic anhydride in 100 ml of acetone, then pour this solution dropwise into the aqueous solution of glycylglycine with magnetic stirring[32]. The turbidity observed during the addition progressively disappears, and subsequently N-methacryloylglycylglycine gradually precipitates out. Keep stirring for 18 h at room temperature. Separate the precipitate by filtration and dry under vacuum the white powder obtained (32.5 g, 81 % yield). The compound decomposes without melting above 200°.

The structure of this monomer was confirmed by elemental analysis (C, H, N, O) and by the NMR spectrum (DMSO-d6): δ 8 ppm (–NH–); δ 5.75 ppm (s) (H–C=); δ 5.35 ppm (H–C=); δ 3.75 ppm (d) ($-CH_2-$); δ 1.9 ppm (s) ($-CH_3$).

Preparation of crosslinked copolymers in the form of beads

The copolymerization of the two monomers prepared above to give beads is based on the method described by Uriel *et al.*[33] for the preparation of mixed polyacrylamide–agarose gels. It consists in preparing an aqueous solution of agarose, acrylamide, N,N'-methylenebisacrylamide and the sodium salt of one of the two previous acid monomers.

Equilibrate the temperature at 50°, add the catalysts and emulsify the solution in vegetable oil[33]. After stirring for 30 min, time required for the polymerization of the acrylic monomers, cool the emulsion and recover the beads by decantation. Wash the gel thoroughly with water and sieve it in order to select the beads between 60 and 140 μm in diameter.

Numerous attempts were necessary in order to establish the optimal conditions for copolymerization in emulsions of the various monomers. If the acid monomers are used as such, the resulting copolymers have the shape of clusters of spherical particles, the size of which depends on the speed of stirring. This result indicates that during the copolymerization, the acid monomer used forms with the TEMED (co-catalyst of the reaction) ammonium salts, which probably alter the stability of the emulsion.

Subsequently, we obtained very regular beads by using the sodium salts of the monomers Ac.N-A-6-H or N-MGG. Under such conditions, copolymerization is carried out with a conversion ranging from 40 to 90%.

Determination of the free carboxyl groups on the gels obtained

The amount of carboxyl groups on the gel was determined, the volume of gel on which the determination is based being the volume in millilitres measured after decanting the gel in aqueous suspension. The methods used for the determination of the acid groups were those described by Inman and Dintzis[34] and Yon and Simmonds[35]. These methods are based on acid–base potentiometric titration on the gel, either directly or indirectly.

RESULTS

Copolymers of 6-acrylamidohexanoic acid

We carried out several polymerizations as mentioned above, varying each time the amounts of reagents in the reaction medium. This allowed us to determine the optimal conditions for preparing the copolymers required bearing variable amounts of carboxyl groups (see Table I). In each instance we obtained regular beads, perfectly spherical and with a diameter ranging from 50 to 200 μm before sieving. The agarose present in the reaction medium is not likely to play a part in the process of polymerization. Its purpose is to give the copolymer a wide mesh and a mechanically resistant structure. The acrylic copolymer is probably regularly distributed inside this network, as is the case with the gels used in the permeation chromatography of polyacrylamide–agarose[33,36,37].

In Table I, we report a few results obtained by varying the concentrations of monomers and cross-linking agent. The results indicate that the amount of carboxyl groups present in the gel is proportional to the amount of functionalized monomers involved in the initial solution, as shown in Fig. 1. Hence, by operating with very precise conditions, it should be possible to obtain reproducibly a gel copolymer con-

TABLE I

YIELD OF COPOLYMERIZATION OF 6-ACRYLAMIDOHEXANOIC ACID (4% CONCENTRATION IN AGAROSE)

Acrylamide concentration (%)	*BIS concentration (%)*	*Ac.N-A-6-H concentration (%)*	*Ac.N-A-6-H (μmole per ml of solution)*	*CO_2H (μmole per ml of gel)*	*Conversion (%)*
—	0.26	7.8	451.61	317	70.19
—	0.26	7.8	451.61	280	62.00
1.0	0.26	4.0	216.21	142	65.67
1.0	0.26	2.0	108.10	48	44.30
—	3.0	1.56	84.32	45	53.36
1.5	3.0	1.56	84.32	42.5	50.40
1.5	2.0	0.78	42.16	22.5	53.37
—	2.0	0.4	21.62	13	60.12
—	2.5	0.4	21.62	13	60.12
—	2.0	0.4	21.62	12.5	57.82
—	2.0	0.3	16.21	9.3	57.35
—	2.0	0.2	10.81	7.4	68.45
—	2.0	0.2	10.81	6.8	62.91
—	2.0	0.2	10.81	6.5	51.80
—	2.0	0.2	10.81	6.13	57.71
—	2.0	0.2	10.81	5.9	54.58

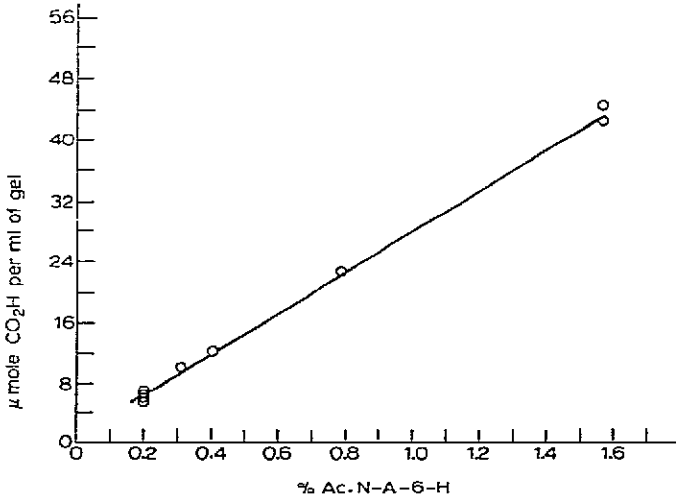


Fig. 1. Concentration of acid groups in the gel *versus* the weight concentration of 6-acrylamidohexanoic acid in the initial solution.

taining a given number of carboxyl groups per volume unit. This can be considered as the first stage towards preparing a gel intended for affinity chromatography, because in our opinion it is the only way of foreseeing the number of carboxyl functions introduced.

Systematic determinations of the carboxyl groups in the gels allowed us to calculate in each instance the conversion rate of the monomers. The conversion rates observed were reproducible within the limits of the operating conditions specified.

Table I and Fig. 2 show the conversion rates of the acid monomer Ac.N-A-6-H, which were calculated without taking into account the interstitial volumes. Fig. 2 shows that whatever the concentration of 6-acrylamidohexanoic acid in the starting solution, the conversion rates observed always range from 50 to 70%. Thus, in order to obtain a gel containing about 20 µmole of carboxyl groups per millilitre,

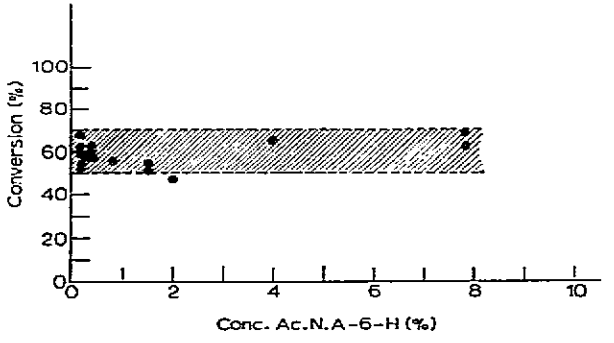


Fig. 2. Conversion of 6-acrylamidohexanoic acid *versus* its weight concentration in the initial solution. The conversion is the proportion of the number of acid groups (µmole) per millilitre of solution to the number of acid groups (µmole) per millilitre of final gel.

theoretically it is advisable to use a solution of monomers containing 0.7% of 6-acrylamidohexanoic acid. By following the conditions of polymerization mentioned above, a gel polymer should be obtained with a number of carboxyl groups varying in the range 19–26 μmole/ml.

N-Methacryloylglycylglycine (N-MGG) copolymers

In the same way as for 6-acrylamidohexanoic acid, numerous copolymerizations of this monomer were effected in emulsion, in the presence of a constant amount of agarose and of variable amounts of co-monomers and crosslinking agent. We obtained gels in the form of very regular spherical beads with diameters ranging from 50 to 200 μm.

In the same way as above, we determined the amount of carboxyl groups on the gel. Table II shows the copolymerization conditions and the results obtained.

Once again it appears that the amount of carboxyl groups is proportional to the amount of N-methacryloylglycylglycine involved (Fig. 3). In the present instance, although the attempts made were less numerous than for the previous copolymer

TABLE II

YIELD OF COPOLYMERIZATION OF N-METHACRYLOYLGLYCYLGLYCINE (WITHOUT ACRYLAMIDE AND WITH AN AGAROSE CONCENTRATION OF 4%)

BIS concentration (%)	*N-MGG concentration (%)*	*N-MGG (μmole per ml of solution)*	*N-MGG (μmole per ml of gel)*	*Conversion (%)*
1.1	11	550	232	42
2	2	100	62	62
2	0.8	40	26	65
2	0.2	10	7.5	75
2	0.2	10	8.5	85
1.5	0.2	10	8.9	89

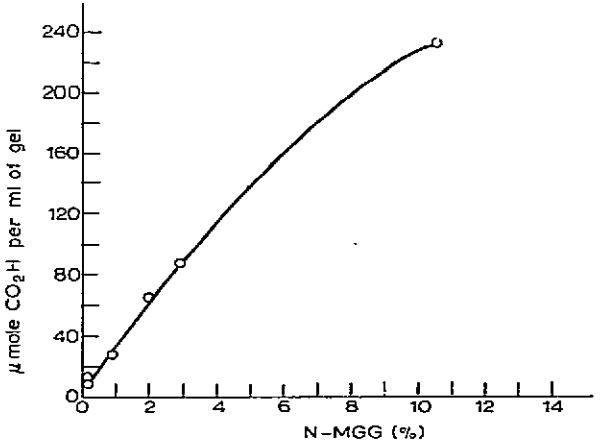


Fig. 3. Concentration of acid groups in the gel *versus* the weight concentration of N-methacryloylglycylglycine (N-MGG) in the solution

(which bears hydrophobic side-chains), we noticed that the conversion rates did not follow the same rule. Indeed, we observed a nearly quantitative transformation at low concentrations (0.2%) of the monomer. As the concentration of the monomer increased, the conversion rate diminished (Fig. 4), whereas it remained more or less constant in the previous instance. In addition, Fig. 4 indicates that for concentrations of monomer above 8–9%, the conversion rate should remain constant at about 40–50%. Fig. 5 indicates the shape of the beads.

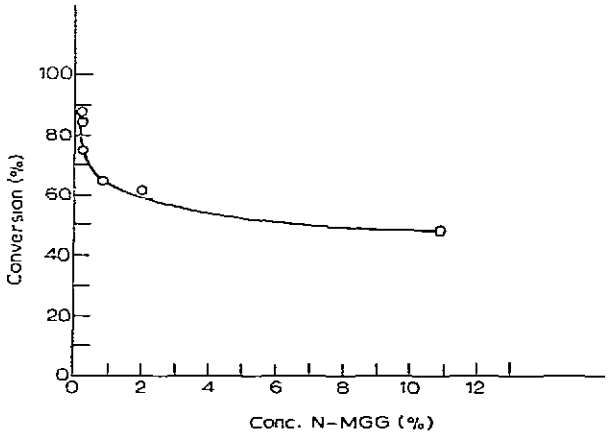


Fig. 4. Conversion of N-methacryloylglycylglycine (N-MGG) *versus* its weight concentration in the initial solution.

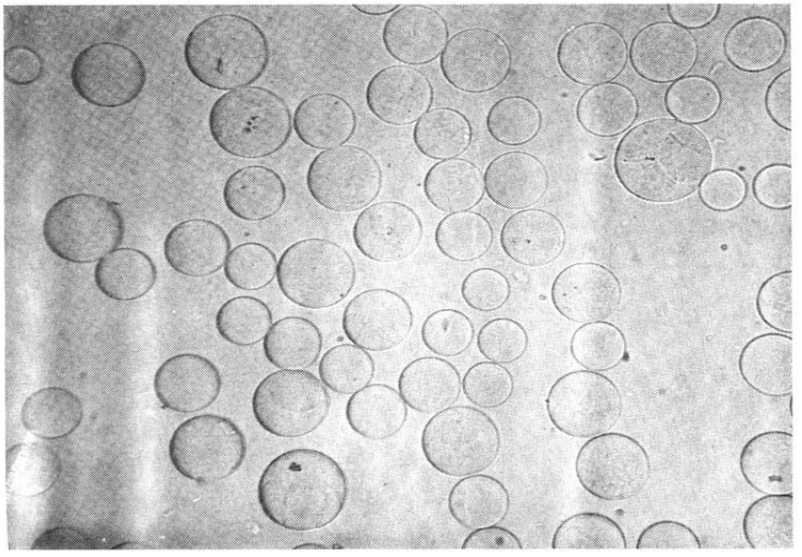


Fig. 5. Copolymer beads corresponding to Table II, last row: agarose (4%)–N,N'-methylenebisacrylamide (2%)–N-methacryloylglycylglycine (0.2%). Magnification: × 100.

DISCUSSION

The mastery of the chemical reactions allowing the reproducible synthesis of a gel, carrying carboxyl groups distant from the matrix, proved to be one of the major problems when developing carriers for affinity chromatography, which is why we studied the first principles of the problem by first preparing acrylic monomers that we subsequently copolymerized. We aimed to obtain a copolymer with such mechanical resistance that it would compare favourably with most of the chromatographic carriers that are currently available. We decided to carry out our copolymerizations in emulsion in order to obtain gels with the shape of porous, resistant, spherical beads.

We chose acrylic acid monomers (N-MGG and Ac.N-A-6-H) for obvious reasons. Acid monomers in which the CO_2H function is far removed from the acrylic double bond are required[15]. The monomer must be easily prepared, soluble in water and insoluble in vegetable oils (at least in the form of the sodium salt), and it must be easily copolymerized with acrylamide and N,N-methylenebisacrylamide. Moreover, we took into account recent studies in affinity chromatography which generally recommend the use of 6-aminohexanoic acid as a spacer arm[38], probably because of its availability and in spite of some drawbacks such as its high hydrophobicity.

According to some workers[39], the use of a spacer arm of this type, with a polymethylenic chain, deprives chromatography of its specific character by superposing, upon affinity proper, complex association phenomena in which hydrophilic and hydrophobic bonds intervene. Further, it is sometimes necessary for these various types of association to occur simultaneously in order to be able to achieve satisfactory separations in this way[1].

It was in the light of these considerations that we undertook the synthesis of 6-acrylamidohexanoic acid. The relative structural simplicity of this compound makes it possible to obtain it in large amounts. We also synthesized a similar, although more hydrophilic monomer, N-methacryloylglycylglycine, in which the acrylic double bond is separated from the carboxyl group by a dipeptidic chain likely to show affinity for the proteins.

We undertook a comparative study of the advantages and drawbacks resulting from the use of copolymers derived from 6-acrylamidohexanoic acid (a carrier with a hydrophobic spacer arm) and N-methacryloylglycylglycine (a carrier with a hydrophilic spacer arm). This study, which deals with the separation of animal and vegetable proteins, will be published in a separate paper[40].

When preparing these copolymers, our long experience of mixed gels (Ultrogels AcA)[33,36,37] helped us in choosing the experimental conditions likely to provide useful results. Thus, we always prepared functionalized copolymers in the presence of agarose, which gives the gel beads the rigidity required for an optimal use in column.

The results obtained show that our method makes it possible to develop a whole range of gels with the number of carboxyl groups required. However, in affinity chromatography the optimal number of functional groups likely to immobilize the ligand is rather low, and generally ranges from 5 to 15 μmole per millilitre of deposited gel. This is important as this range imposes a very low concentration of functionalized acrylic monomer (about 0.2% whether the hydrophobic or the hydrophilic monomer is concerned). Under such conditions, the consumption of relatively

expensive acrylic product is reduced to the minimum. Also, it was with these concentration rates that the reproducibility of our experiments was optimal and that the results of polymerization could be predicted most easily.

We believe that we have partly solved the problems of reproducibility in the preparation of carriers for affinity chromatography. This new technique involves the use of less toxic and dangerous chemicals than the reagents currently used, especially cyanogen bromide in aqueous[7] or organic[41,42] solutions.

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